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A Japanese Encephalitis Virus Peptide Present on Johnson Grass Mosaic Virus-Like Particles Induces Virus-Neutralizing Antibodies and Protects Mice against Lethal Challenge

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Protection against Japanese encephalitis virus (JEV) is antibody dependent, and neutralizing antibodies alone are sufficient to impart protection. Thus, we are aiming to develop a peptide-based vaccine against JEV by identifying JEV peptide sequences that could induce virus-neutralizing antibodies. Previously, we have synthesized large amounts of Johnson grass mosaic virus (JGMV) coat protein (CP) in *Escherichia coli* and have shown that it autoassembled to form virus-like particles (VLPs). The envelope (E) protein of JEV contains the virus-neutralization epitopes. Four peptides from different locations within JEV E protein were chosen, and these were fused to JGMV CP by recombinant DNA methods. The fusion protein autoassembled to form VLPs that could be purified by sucrose gradient centrifugation. Immunization of mice with the recombinant VLPs containing JEV peptide sequences induced anti-peptide and anti-JEV antibodies. A 27-amino-acid peptide containing amino acids 373 to 399 from JEV E protein, present on JGMV VLPs, induced virus-neutralizing antibodies. Importantly, these antibodies were obtained without the use of an adjuvant. The immunized mice showed significant protection against a lethal JEV challenge.

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus responsible for acute encephalitis in humans, with fatality rates ranging from 20% to as high as 50%. The virus is active in a vast geographic area that includes India, China, Japan, and almost all of Southeast Asia. As many as 50,000 cases of JEV infection are reported from these areas every year, of which about 10,000 result in fatality. A high proportion of survivors have serious neurologic and psychiatric sequelae (34). A mouse brain-grown, formalin-inactivated vaccine is available internationally that has many limitations; it is expensive to produce, does not provide long-term immunity, and may cause allergic reactions because of the inclusion of murine encephalogenic basic protein or gelatin stabilizer (1, 20, 25, 27). Thus, there is a need to produce an alternate vaccine that may be safer and cheaper.

JEV infection of host cells produces three structural and seven nonstructural proteins. One of these, the E protein, is the major envelope protein of the virion. This protein is believed to play an important role in a number of processes, including viral attachment, membrane fusion, and entry into the host cell. In response to JEV infection, the host produces virus neutralizing antibodies and cytotoxic T cells (CTLs). The principal target for the neutralizing antibodies is the E protein. It has been shown that protection against JEV infection is mainly antibody dependent, and virus-neutralizing antibodies alone are sufficient to impart protection (10, 18). Peptide(s) from JEV E protein that forms the virus-neutralizing epitope(s) could, therefore, be used for inducing JEV-neutralizing antibodies.

Recent findings suggest that presentation of peptides in a highly ordered aggregate form can result in enhanced immune responses (12). Johnson grass mosaic virus (JGMV) coat protein (CP) has been shown to self-assemble to form rod-shaped virus-like particles (VLPs) even in the absence of the viral RNA (7). In the present study, peptide sequences chosen from JEV E protein, with a potential to induce anti-JEV neutralizing antibodies, were fused to the C terminus of JGMV CP by using recombinant DNA methods. The recombinant fusion CP containing JEV peptide sequence, synthesized in *Escherichia coli*, was shown to autoassemble and form VLPs. Immunogenicity studies of these VLPs in experimental animals showed that recombinant VLPs presented the peptide sequences efficiently to the immune system without the need for an adjuvant, and a vigorous anti-peptide and anti-E immune response was generated that cross-reacted with JEV. Mice immunized with VLPs presenting a 27-amino-acid peptide from JEV E protein (amino acids 373 to 399) generated JEV-neutralizing antibodies, and these mice were protected against a lethal JEV challenge.

MATERIALS AND METHODS

Virus and cells. The JaOAr strain of JEV was used in these studies. The virus was grown in porcine kidney (PS) cells obtained from the National Centre for Cell Sciences, Pune, India. PS cells were grown in Eagle minimal essential medium supplemented with 10% fetal calf serum.

Construction of *E. coli* expression plasmids for the synthesis of JGMV CP fused to JEV peptides. The cDNA encoding JGMV CP was placed under the control of the bacteriophage T7 promoter in *E. coli* expression vector pVex to generate pVexCP. This construct was shown to synthesize large amounts of JGMV CP in *E. coli*. The bacterially synthesized CP autoassembled to form VLPs (26). Figure 1 shows the map of the *E. coli* expression vector pVexCP and its unique restriction sites used in the present study. The unique *Pst*I site, 42 nucleotides upstream of the CP stop codon, and the unique *Hin*dIII site in the vector, downstream of the CP sequence, were used for the fusion of the JEV peptide sequences to the CP. Thus, 14 amino acids at the C terminus of the CP were swapped with JEV peptides.

The cDNA encoding the 27-amino-acid peptide A (Table 1) was amplified by

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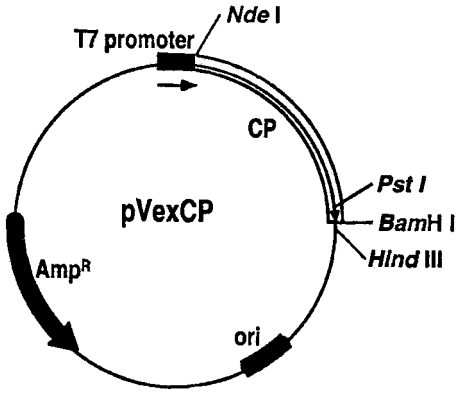

FIG. 1. Map of the expression vector pVexCP. The *E. coli* expression vector pVexCP was made by cloning the modified DNA encoding JGMV CP downstream of the bacteriophage T7 promoter between the *Nde*I and the *Bam*HI sites of vector pVex11. The unique *Pst*I site, 42 nucleotides upstream of the CP stop codon, is marked. JEV peptide encoding DNA was fused directly to CP cDNA by swapping the *Pst*I and the *Bam*HI fragment. In the case of the linker-mediated fusion of JEV peptide to CP, the linker-encoding DNA replaced the *Pst*I and the *Bam*HI fragment of pVexCP and the JEV peptide encoding DNA was then placed between the *Bam*HI and the *Hin*dIII sites. All fusion constructs were sequenced to ensure the presence of the desired peptide- and/or the linker-encoding DNA in the correct reading frame.

the reverse transcription-PCR (RT-PCR) with the oligonucleotides SV218 (*CT GCAG*AGATGGAACCCCCCTTC) and SV219 (*AAGC***TTATTA**GGCTTTGT GCCAATGGTG) with JEV RNA used as the template. SV218 contained *Pst*I site (in italics), whereas SV219 contained *Hin*dIII site (in italics), and the complement of two stop codons in tandem (in boldface). The JEV genome sequence within these oligonucleotides has been underlined. The 96-nucleotide RT-PCR product was digested with *Pst*I and *Hin*dIII and ligated with *Pst*I- and *Hin*dIII-digested pVexCP to generate expression vector pVexCP-A that was used for the synthesis of CP fused to JEV peptide A (CP-A) in *E. coli*. For the synthesis of CP fused to JEV peptide B (CP-B) or peptide C (CP-C), expression vector pVexCP-B or pVexCP-C was generated by ligating the *Pst*I- and *Hin*dIII-digested pVexCP with an adapter DNA encoding the JEV peptide. The adapter for the peptide B was made by annealing the oligonucleotides SV253 (GGGAGG GGAGACAAGCAGATCAACCACCATTGGCACAAAGCTTA) and SV254 (AGCTTAAGCTTTGTGCCAATGGTGGTTGATCTGCTTGTCTCCCCTCCC TGCA). The adapter for the peptide C was made by annealing the oligonucleotides SV236 (GATAACCATGGGAATTATTCAGCGCAAGTTGGGGCGT CCCAGTA) and SV237 (AGCTTACTGGGACGCCCCAACTTGCGCTGAA TAATTCCCATGGTTATCTGCA). JEV genome sequences within these oligonucleotides are underlined. These adapters also contained a translation stop codon at the end of the JEV sequence.

In another set of fusions, a 15-amino-acid linker encoding (Gly-Gly-Gly-Gly-Ser)$_3$ was inserted between the JGMV CP and the JEV peptide sequence. For this, the last 14 amino acids of the CP were swapped with the peptide linker by inserting the DNA encoding the linker in between the *Pst*I and the *Bam*HI sites of pVexCP to generate pVexCP^L. The linker DNA was obtained by annealing the synthetic oligonucleotides SV232 (GGTGGCGGTGGAAGTGGTGGCGG TGGAAGTGGTGGCGGTG) and SV233 (GATCCACCGCCACCACTTG CACCGCCACCACTTCCACCGCCACCTGCA). The expression vectors pVexCP^L-A (for the synthesis of CP fused to JEV peptide A through the linker, CP^L-A) and pVexCP^L-D (for the synthesis of CP^L-D) were made by inserting JEV peptide-encoding RT-PCR product between the *Bam*HI and the *Hin*dIII sites of pVexCP^L. The JEV peptide A encoding DNA was RT-PCR amplified from JEV RNA with the oligonucleotides SV257 (*GGA TCC*GAGATGGAACCCCCCTTC) and SV219. The JEV peptide D encoding DNA was RT-PCR amplified from JEV RNA with the oligonucleotides SV285 (*GGATCC*ATGTGTACAGAAAAATTCTCGTTC) and SV286 (*AA GCTT*ATTATCCAGCTTTGTGCCAATGGTGGTTGAT). Here, the sequence in italics denotes a restriction site sequence used for cloning purposes, whereas the underlined sequence relates to the JEV genome. Oligonucleotides SV219 and SV286 contained stop codons also. Expression vectors pVexCP^L-B (for the synthesis of CP^L-B) and pVexCP^L-C (for the synthesis of CP^L-C) were made by inserting the JEV peptide-encoding adapter DNA between the *Bam*HI and *Hin*dIII sites of pVexCP^L. The adapter encoding the peptide B was produced by annealing oligonucleotides SV255 (GATCCGG GAGGGGAGACAAGCAGATCAACCACCATTGGCACAAAGCTTA) and SV256 (AGCTTAAGCTTTGTGCCAATGGTGGTTGATCTGCTTGT CTCCCCTCCCG). The adapter DNA encoding peptide C was produced by annealing oligonucleotides SV234 (GATCCAACCATGGGAATTATTCAG CGCAAGTTGGGGCGTCCCAGTA) and SV235 (AGCTTACTGGGACG CCCCAACTTGCGCTGAATAATTCCCATGGTTG). The JEV-related sequences within these oligonucleotides are underlined. Oligonucleotides SV256 and SV235 contained the translation stop codons also. The presence of the desired JEV sequence and the continuity of the open reading frame were established by nucleotide sequencing of the plasmid constructs.

JGMV CP synthesis in *E. coli*. The *E. coli* expression plasmid containing the desired DNA sequence under the control of the bacteriophage T7 promoter was transferred to *E. coli* BL21(DE3). The transformed bacteria were inoculated into Luria broth containing ampicillin. The culture was allowed to grow until it reached an absorbance at 600 nm of 0.5. At this stage, the culture was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 37°C for 3 h. The cell pellet was lysed with loading dye (50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% sodium dodecyl sulfate [SDS], 0.1% bromophenol blue, 10% glycerol) and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). To check for the protein synthesis, the gel was stained with 0.25% Coomassie brilliant blue R-250 dye (Sigma) and destained with a methanol-water-acetic acid mixture (45:45:10).

Purification of the VLPs. *E. coli* cells synthesizing JGMV CP or the fusion protein were pelleted, suspended in 10 mM Tris-HCl (pH 7.4), and sonicated (Branson sonifier). The suspension was centrifuged at 20,000 × *g* for 10 min at 4°C. The supernatant was then loaded onto a 10 to 40% discontinuous sucrose gradient and centrifuged in an ultracentrifuge (Sorvall) at 50,000 rpm for 3 h in a TST60.4 rotor at 4°C. The pellet was resuspended in 10 mM Tris-HCl (pH 7.4) and again layered onto a discontinuous sucrose gradient as described above, followed by centrifugation at 50,000 rpm for 1 h in a TST60.4 rotor at 4°C. The pellet containing the VLPs was resuspended in 10 mM Tris-HCl (pH 7.4). The protein content of the purified VLP preparation was estimated by the Bradford method by using Bio-Rad protein assay kit (Bio-Rad).

Mice immunization and challenge. Groups of four to six FVB/J mice (6 weeks old) were injected subcutaneously with appropriate amount of VLPs that contained 10 μg of JEV peptide sequence. The VLPs were suspended in saline and used without any adjuvant. Primary immunization was followed by two booster doses given 3 and 4 weeks later. The animals were bled retro-orbitally, and sera

TABLE 1. JEV peptide sequences chosen for presentation on recombinant VLPs[a]

Peptide	Amino acid sequence	Location in the JEV E protein (amino acid range)
A	EMEPPFGDSYIVVGRGDKQINHHWHKA	373–399
B	GRGDKQINHHWHKA	386–399
C	NHGNYSAQVGASQ	151–163
D	MCTEKFSFAKNPADTGHGTVVIELSYSGSDGPCKIPIVSVASLNDMTPVGRLVT VNPFVATSSANSKVLVEMEPPFGDSYIVVGRGDKQINHHWHKAG	303–400

[a] The amino acid sequences of the peptides chosen from the E protein of JEV, for presentation on JGMV VLPs are given. The location of the peptide sequences within the amino acid sequence of the JEV E protein has been indicated elsewhere (33).

were stored at −70°C. For the challenge experiment, mice were injected intracerebral with 10 50% lethal doses of JEV.

ELISA. An enzyme-linked immunosorbent assay (ELISA) plate was coated with 1 μg of peptide or JEV E protein in 0.2 M carbonate buffer (pH 9.6) overnight at 4°C. The peptides were synthesized at the NII peptide facility or were obtained commercially. The JEV E protein was produced in *E. coli* and purified as described by Kaur et al. (8). For determination of anti-JEV antibodies, the ELISA plate was coated with C6/36 cell-grown JEV as described previously (22). The ELISA plate was washed three times with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T), and the wells were blocked with 1% lactogen in PBS-T at 37°C for 2 h. The plate was washed with PBS-T three times and then incubated with 100 μl of diluted mice sera per well at 37°C for 1 h. The plate was washed again thrice with PBS-T, and 100 μl of anti-mouse antibody conjugated to horseradish peroxidase (Dako), diluted 1:2000, was added per well, followed by incubation at 37°C for 1 h. The antibody conjugate was removed by washing the plate three times with PBS-T. The plate was then incubated in the dark with 100 μl per well of the substrate *o*-phenylenediamine dihydrochloride (0.5 mg/ml) prepared in citrate buffer (1% citric acid, 1.46% disodium hydrogen phosphate) at room temperature for 10 min. The reaction was stopped by adding 50 μl of 5 N sulfuric acid per well. The absorbance was read at 492 nm in an ELISA reader (Anthos Labtech HT2).

JEV neutralization assay. Diluted mouse sera were heat inactivated at 56°C for 30 min and incubated with ~100 PFU of JEV at 37°C for 1 h. The virus titers were then determined by plaque formation on PS cell monolayers (32). The percent neutralization was calculated from the number of plaques obtained in the presence of the preimmune and the postimmunization sera. The reciprocal of the highest serum dilution giving at least 50% neutralization was regarded as the JEV neutralization titer.

RESULTS

Selection of peptide sequences with potential to give JEV-neutralizing antibodies. Most of the immune responses against JEV are directed against the E protein, including the virus-neutralizing antibodies. A 27-amino-acid peptide spanning residues 373 to 399 of the JEV E protein fused to glutathione *S*-transferase was shown to induce neutralizing antibodies in mice when it was administered with a strong adjuvant (28). We chose this peptide sequence (peptide A) to be presented on the JGMV VLPs. Another peptide sequence that was chosen for presentation on the JGMV VLPs was a subset of the 27-amino-acid sequence; it contained residues 386 to 399 of JEV E protein (peptide B). A third peptide contained residues 151 to 163 of the E protein (peptide C). This peptide was chosen as a potential B-cell epitope based on the predicted three-dimensional structure of JEV E protein (9). In addition to being hydrophilic and charged, this region contained a potential glycosylation site and hence a putative epitope. We also chose a 98-amino-acid sequence (peptide D) from the E protein (from methionine 303 to glycine 400) for presentation on JGMV VLPs. The choice for this region was based on the observation that a 94-amino-acid peptide from JEV E protein (amino acids 303 to 396) contained binding sites for 10 anti-JEV monoclonal antibodies that neutralized the virus activity in vitro (14). In addition, some of these antibodies passively protected mice from a fatal virus challenge, and hence the peptide had the potential to induce virus-neutralizing antibodies. The amino acid sequences of these peptides and their location in the JEV E protein is given in Table 1.

Fusion of JEV peptides to JGMV CP. We have previously synthesized large amounts of JGMV CP in *E. coli* by using expression vector pVexCP that contained a modified CP-encoding DNA under the control of the bacteriophage T7 promoter (26). The bacterially synthesized modified JGMV CP was shown to autoassemble to form VLPs. Using the recombinant DNA technology, the last 14 amino acids at the C-terminal of JGMV CP were swapped with JEV peptides with or without a 15-amino-acid peptide linker $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$ in between the CP and the JEV peptide. Figure 2 shows synthesis of various fusion proteins in *E. coli*. The levels of the CP fusion protein in the *E. coli* lysates were high, although variations were seen at times. For example, the level of CP^L-A was low in a particular experiment (as shown in Fig. 2), but it was as high as for other fusion proteins in another experiment. The mobilities of the fusion proteins in the SDS-PAGE analyses were commensurate with their predicted molecular masses. The N-terminal amino acid sequence of fusion proteins matched that for JGMV CP. The fusion proteins could be specifically Western blotted from the *E. coli* lysates with rabbit anti-CP and mouse anti-JEV sera (data not shown).

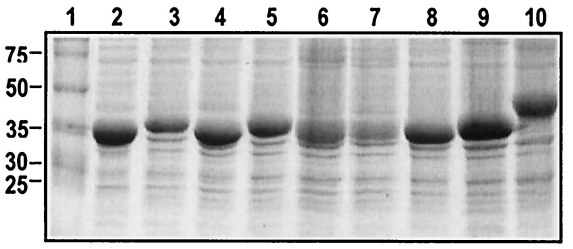

FIG. 2. Synthesis in *E. coli* of JGMV CP fusion proteins containing JEV peptide. Expression of various plasmid constructs containing fusion of CP DNA with DNA-encoding JEV peptides A, B, C, and D, with or without the linker, was studied in *E. coli* BL21. The cell lysates were separated on a SDS–12% PAGE gel. Shown above is the Coomassie blue-stained gel. Lane 1, protein size markers in kilodaltons (indicated at the left); lane 2, *E. coli* lysate containing CP; lane 3, *E. coli* lysate containing CP-A; lane 4, *E. coli* lysate containing CP-B; lane 5, *E. coli* lysate containing CP-C; lane 6, *E. coli* lysate containing CP^L; lane 7, *E. coli* lysate containing CP^L-A; lane 8, *E. coli* lysate containing CP^L-B; lane 9, *E. coli* lysate containing CP-^{L}C; lane 10, *E. coli* lysate containing CP^L-D.

The fusion protein produced in *E. coli* autoassembled to form VLPs. Figure 3 shows electron micrographs of an *E. coli* cell containing the VLPs formed by the CP fused to the JEV peptide A (CP-A); these were ~20 nm thick. The VLPs could be conveniently purified by sucrose gradient centrifugation, and they were readily visible with an electron microscope. The purified VPLs were substantially free from contaminating proteins, and they contained almost pure fusion protein when analyzed by SDS-PAGE.

Immunization of animals with VLPs presenting JEV peptides. FVB/J mice in a group of four to six animals each were immunized with recombinant VLPs containing 10 μg of JEV peptide A, B, C, or D. The VLPs in saline were injected subcutaneously, without any adjuvant. Booster doses were given on days 21 and 28 postimmunization. The booster dose in saline had recombinant VLPs that contained 10 μg of JEV peptide sequence. Mice sera were assayed for anti-peptide antibodies with the cognate peptide as the capture antigen in ELISA. Anti-peptide antibodies could be found in immunized mice after the primary immunization, and these antibody levels increased substantially after the booster doses were given (Fig. 4). In the case of peptide A, the antibody response was significantly higher when the peptide was presented on the VLPs by using a linker. This was also true in the case of peptide C,

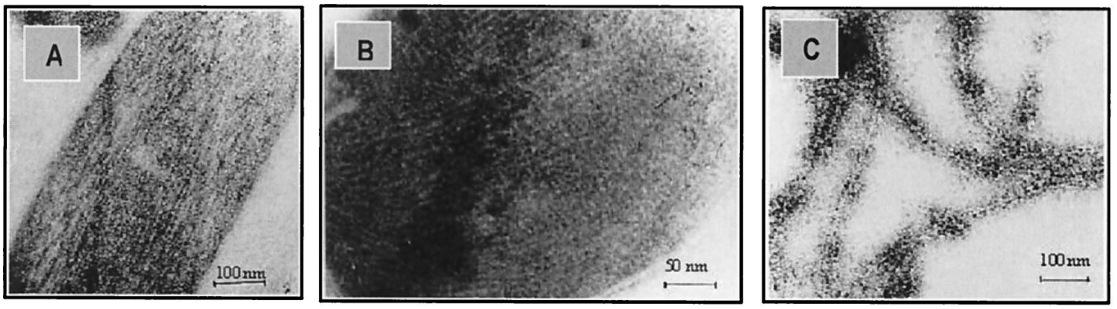

FIG. 3. VLP formation in *E. coli* by JGMV CP fused with JEV peptide A. pVexCP-A transformed *E. coli* BL21 cells were grown and induced for the CP-A synthesis with 1 mM IPTG at 37°C. (A to C) Transmission electron micrographs of a section of *E. coli* cell synthesizing JGMV CP-A seen at ×26,000 magnification (A), a section of *E. coli* cell synthesizing JGMV CP-A seen at ×52,000 magnification (B), and an *E. coli* cell lysate containing JGMV CP-A seen at ×105,000 magnification (C). Long, flexuous VLPs are visible in *E. coli* cells and in the cell lysate.

although the difference did not appear to be as pronounced as in the case of peptide A. In the case of peptide B, however, the difference between the antibody responses induced by CP-B and CP^L-B did not appear to be significant. The antibody response of mice immunized with CP^L-D was assayed by using JEV peptide A for capturing the antibodies in ELISA, since peptide A sequence was a subset of the peptide D sequence. Figure 4 shows that the large peptide D was poorly immunogenic compared to the smaller peptides, including peptide A, whose amino acid sequence is a part of the peptide D sequence. The low antibody titers in the case of CP^L-D-immunized mice were not due to the use of a smaller peptide (peptide A) as the capture antigen in ELISA since titers were low even when JEV E protein was used as the antigen in ELISA (see below).

The immunized mice sera were subsequently assayed for the anti-E antibodies by using purified JEV E protein as the antigen in the ELISA. Recombinant VLPs presenting JEV peptide A, B, C, or D induced antibodies that reacted with JEV E protein (Fig. 5). The anti-E immunogenicity pattern induced by the recombinant VLPs was similar to that described above for the anti-peptide antibodies. Thus, CP^L-A was most immunogenic, followed by CP^L-B and CP^L-C, whereas CP^L-D showed very poor immunogenicity. In rats also, the recombinant VLPs were immunogenic without the use of an adjuvant and the pattern of immunogenicity of different VLPs was similar to that seen in mice (data not shown). The sera from the recombinant VLP-immunized animals also reacted with JEV, as seen by immunofluorescence on JEV-infected cells (data not shown).

The ELISA results shown above indicated that peptide A-containing VLPs were highly immunogenic in mice and that CP^L-A-containing VLPs were more immunogenic than CP-A-containing VLPs. In a separate experiment, the immunogenic-

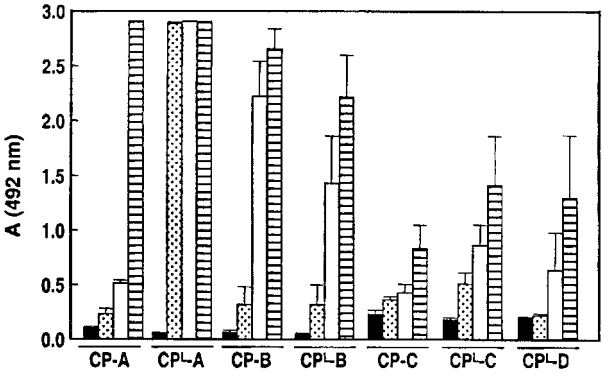

FIG. 4. Anti-peptide antibody response in mice. Mice were immunized with the recombinant VLPs presenting JEV peptides. Three weeks later a booster immunization was given that was followed by another booster given 4 weeks postimmunization. Mice were bled at various time points. Serum samples were diluted 1:50, and anti-peptide antibodies were analyzed by ELISA with the cognate peptide. For the ELISA of the bleeds obtained from mice immunized with CP^L-D, peptide A was used as the capture antigen. The mean ELISA absorbance values for different groups of mice are shown. The solid bars represent preimmunization bleeds, the dotted bars represent bleeds obtained 3 weeks postimmunization, the open bars represent bleeds obtained 1 week after the first booster, and the horizontally lined bars represent bleeds obtained 1 week after the second booster.

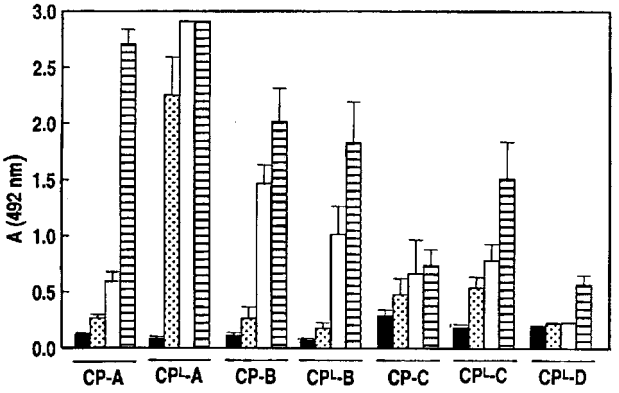

FIG. 5. Anti-JEV E protein antibody response in mice. Mice were immunized with the recombinant VLPs presenting JEV peptides. Three weeks later a booster immunization was given that was followed by another booster given 4 weeks postimmunization. Mice were bled at various time points. Serum samples were diluted 1:50, and anti-E antibodies were analyzed by ELISA with the E protein of JEV for the antibody capture. The mean ELISA absorbance values for different groups of mice are shown. The solid bars represent preimmunization bleeds, the dotted bars represent bleeds obtained 3 weeks postimmunization, the open bars represent bleeds obtained 1 week after the first booster, and the horizontally lined bars represent bleeds obtained 1 week after the second booster.

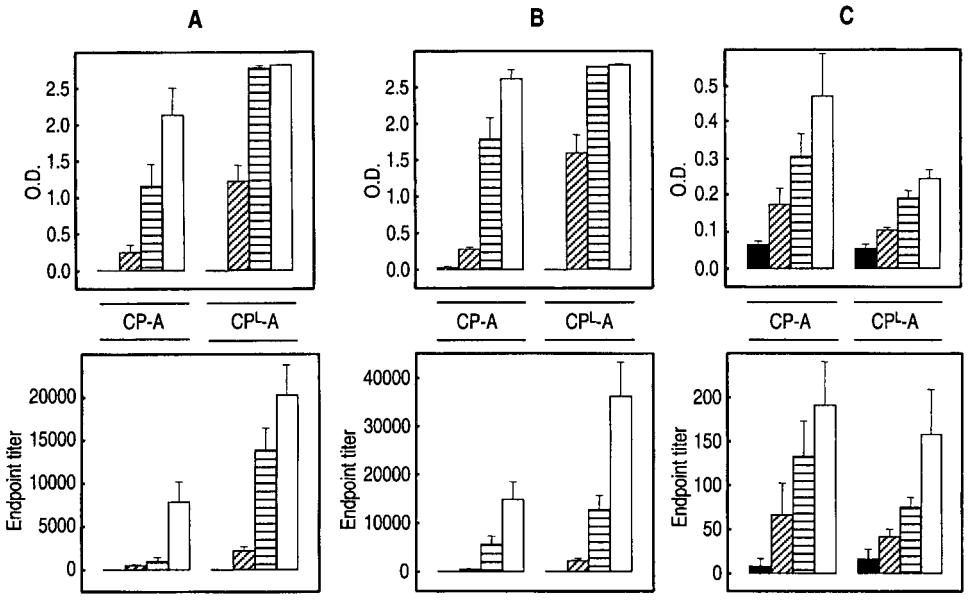

FIG. 6. Endpoint ELISA titers of mice sera. Mice were immunized with the recombinant VLPs presenting JEV A peptide as CP-A or CP^{L}-A. Three weeks later, a booster immunization was given that was followed by another booster given 4 weeks postimmunization. Mice were bled at various time points. Serum samples were diluted 1:50, and anti-peptide (A), anti-E (B), and anti-JEV (C) antibodies were assayed by ELISA with peptide A, JEV E protein, or JEV virions for the antibody capture, respectively. The upper panels show the mean ELISA optical densities (O.D.). The serum samples were serially diluted and assayed for the ELISA endpoint titers. The reciprocal of the highest sera dilution that gave an optical density at least twice of that given by the negative control was considered the ELISA endpoint. The lower panels show the mean endpoint titers. The solid bars represent preimmunization bleeds, the diagonally lined bars represent bleeds obtained 3 weeks postimmunization, the horizontally lined bars represent bleeds obtained 1 week after the first booster, and the open bars represent bleeds obtained 1 week after the second booster.

ity of these VLPs was compared by determining the endpoint titers of individual serum samples from the groups of immunized mice. Figure 6 shows that, compared to CP-A-containing VLPs, CP^{L}-A-containing VLPs induced ~2.6-fold-higher anti-peptide antibody titers and ~2.3-fold-higher anti-E antibody titers. Interestingly, CP^{L}-A-containing VLPs induced lower anti-JEV titers than CP-A-containing VLPs. Although this difference in anti-JEV titers was not statistically significant, it was reproducible.

JEV neutralization by anti-peptide antibodies. Peptides A, B, C, and D fused to JGMV CP induced anti-peptide antibodies in mice that also reacted with JEV. A neutralization assay was carried out to find out the ability of the antibodies raised against the peptides to bind to the virus particles and prevent them from infecting the cells in vitro. The JEV neutralization titers (50% neutralization endpoint) for anti-CP-A and anti-CP^{L}-A sera were 1:40 and 1:20, respectively. The neutralization titers were <1:10 for all other sera, including the ones obtained from mice immunized with VLPs containing just the CP.

Mice protection studies. CP-A- and CP^{L}-A-immunized mice showing JEV neutralization activity were challenged with 10 times the 50% lethal dose of JEV given intracerebrally. All mice (n = 6) that were immunized with CP-A survived, whereas four of six mice immunized with CP^{L}-A survived. None of the unimmunized mice (n = 6) survived the challenge. Thus, CP-A-immunized mice showed 100% protection, whereas CP^{L}-A-immunized mice showed 67% protection (Fig. 7). It should be noted that all mice immunized with VLPs containing just the CP died of the virus challenge.

DISCUSSION

The flavivirus E protein, having an important role in establishing viral infection in the host cell, is the major target for virus-neutralizing antibody response (15). JEV infection in a host produces both the virus-neutralizing antibodies and the CTLs. It has, however, been shown that protection against JEV is mainly antibody dependent and that virus-neutralizing anti-

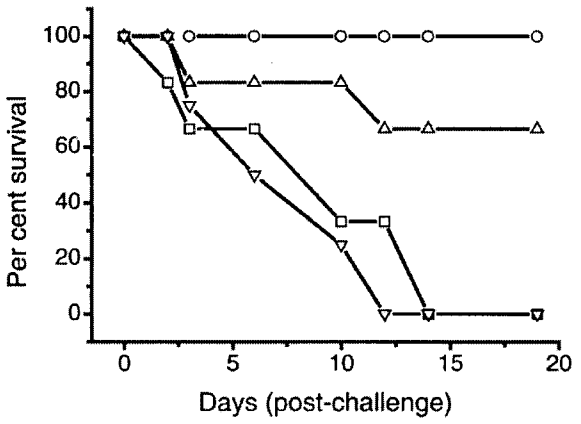

FIG. 7. Mouse survival after challenge. Mice immunized with VLPs containing CP-A, CP^{L}-A, or CP were challenged 2 weeks after the second booster dose with 10 50% lethal doses of JEV given intracerebrally. Mice were observed for mortality. The percentage of surviving mice at a given time point is shown. ○, Mice immunized with VLPs containing CP-A; △, mice immunized with VLPs containing CP^{L}-A; ▽, mice immunized with VLPs containing CP; □, unimmunized mice.

bodies alone are sufficient to impart protection (10, 18). This is also evident from the fact that the formalin-inactivated JEV vaccine, which does not induce CTLs (8), provides protection to vaccinees against JEV. It therefore appears that the B-cell epitopes on JEV E protein are important determinants of protection against virus infection.

As our knowledge of the immune responses to a protein antigen progressed, it became clear that the whole protein was not necessary for raising the immune response, but small segments of protein called the antigenic determinants or the epitopes were sufficient for eliciting the desired immune response. Thus, in recent years a number of peptide sequences have been described for several pathogens that have potential to be used as vaccines (31).

Peptide vaccines have a number of advantages. First, peptide vaccines are easy to produce, since they are well-defined chemical products. They are biologically stable at high temperatures, which makes them useful in tropical regions. Since there is no infectious agent (or nucleic acids) present in the preparation, they are considered safe for human use. Importantly, since peptide vaccines are designed to contain only the desired B- or the T-cell epitopes, they are likely to be free from undesirable amino acid sequences, such as those capable of inducing immunosuppression, molecular mimicry with the host sequences (which could lead to autoimmune disease), and infection-enhancing antibody responses that could have serious vaccination repercussions, especially in the case of flaviviruses (5, 16). A major problem with the peptide vaccines, however, is their poor immunogenicity since short amino acid sequences, on their own, are not very immunogenic. In order to elicit a good immune response, synthetic peptides need to be presented along with a strong adjuvant such as complete Freund adjuvant. The use of such strong adjuvants in humans is considered undesirable. Alternatively, to improve immunogenicity, the peptides could be chemically conjugated to a large carrier protein. However, the process of chemical conjugation is not very reproducible, and uniformity of the peptide density on the carrier protein cannot be ensured. Recent findings show that peptides presented in a particulate form result in enhanced immune responses (12). JGMV CP has been shown to self-assemble to form rod-shaped VLPs even in the absence of the viral RNA (7). A modified CP can be synthesized in high amounts in *E. coli*, in which it self-assembles to form VLPs (26).

The N- and the C-terminal regions of the JGMV CP are surface exposed and thus could be used for the fusion of the foreign peptide sequences (29). In order to get high levels of JGMV CP synthesis in *E. coli*, we made certain changes to the 5′ end of the CP encoding sequence that relieved the CP mRNA translation initiation codon from the stable hairpin-loop structure (26). Fusion of the JEV peptide A to the N terminus of CP was found to cause the reappearance of the hairpin-loop structure around the fusion mRNA translation initiation codon, resulting in no synthesis of CP in *E. coli*. Thus, to maintain the optimal 5′-end sequence of the CP mRNA that was efficiently translated in *E. coli* resulting in high levels of CP synthesis, we avoided fusing the JEV peptide to the N terminus of the CP, and all of the JEV peptide fusions were made to the C terminus. The 14 C-terminal amino acids from JGMV CP were successfully swapped with JEV peptide sequences of various lengths. The fusion protein synthesized efficiently in *E. coli* to high levels. Since the fusion protein autoassembled efficiently to form VLPs, it would appear that the C terminus 14 amino acids of JGMV CP are not necessary for its folding.

The conformation of the peptide is an important determinant of its immunogenicity, and it may determine whether the anti-peptide antibodies would also recognize the native protein from which the peptide was derived. A peptide fused to a large protein such as JGMV CP will have constraints on its conformation imposed by the protein. Thus, to improve chances of producing anti-peptide antibodies capable of recognizing JEV E protein, a flexible linker was used to attach the peptides with the CP in the fusion protein. The flexible linker would provide the peptide freedom to take up a conformation without being affected by the conformation of the CP. Linker sequence composition could have a significant effect on the folding and stability of the fusion protein (24). An ideal linker used to fuse two protein domains should give them flexibility to fold independently, thus retaining their individual activities. We used a linker containing the triple repeat of Gly-Gly-Gly-Gly-Ser residues. This sequence had previously been used effectively to fuse angiogenin and a single-chain antibody against human transferrin receptor without the loss of the antibody binding ability (17). The JGMV CP fused to the JEV peptides through the linker was synthesized in large amounts in *E. coli*, and it assembled efficiently to form VLPs.

The recombinant VLPs containing JEV peptides fused to JGMV CP could be conveniently purified by sucrose gradient centrifugation, and these were used to immunize the experimental animals without the use of an adjuvant. Anti-peptide antibodies were detectable in most of the VLP-immunized mice after the primary immunization, and the level of antibodies increased significantly after the booster dose was given. These results demonstrated the potential of the JGMV VLPs to efficiently present peptide antigen to the immune system and also generate vigorous anti-peptide antibody response without the use of an adjuvant. In fact, anti-peptide antibody titers generated with the recombinant JGMV VLPs were significantly higher than those generated by chemical conjugation of peptides to bovine serum albumin or tetanus toxoid (unpublished results). The JGMV VLP system could thus be useful for the development of other peptide vaccines.

A variation was observed in the mouse antibody response to different JEV peptides presented on the recombinant JGMV VLPs. The antibody titer was very high with peptide A compared to the other three peptides. The anti-peptide antibodies also reacted with JEV E protein and neutralized JEV activity in vitro, indicating that recombinant JGMV VLPs containing JEV peptides were able to produce anti-JEV antibodies. The antibody response to JEV E protein was also highest with peptide A containing VLPs. Interestingly, peptides A and C, present on VLPs with the help of the linker, were generally more immunogenic than when fused to the CP directly at its C terminus. However, this was not the case with peptide B. Nevertheless, it would be advisable to present the peptides on VLPs by using the linker, since this would provide flexibility to the peptide to take conformation(s) independent of the CP.

An interesting observation was made when titers of antibodies raised with VLPs containing the A peptide were measured against the free peptide, the purified JEV E protein, or the

whole virion. Thus, compared to VLPs containing CP-A, the VLPs consisting of CP^{L}-A induced higher levels of anti-peptide and anti-E antibodies but lower titers of anti-JEV antibodies. Interestingly, this result was consistent with a lower level of protection induced by VLPs containing CP^{L}-A than those made up of CP-A. It is possible that the free peptide and the purified E protein, which may be present in several conformations, detected a large variety of antibodies reflecting a higher antibody titer. In the whole virion, however, the sequence pertaining to the A peptide may be present only in a certain conformation, and hence it would react only to a part of the total anti-peptide antibodies, resulting in a lower antibody titer.

The anti-peptide antibodies generated with the VLPs interacted with JEV. Peptide A induced JEV neutralization activity. Peptides B, C, and D induced antibodies with very little, if any, JEV-neutralizing activity. This finding is interesting since the peptide B sequence was a subset of the peptide A sequence; the 14-amino-acid peptide B formed the C terminus of the 27-amino-acid peptide A. It could therefore be argued that the first 13 amino acids of peptide A, representing amino acids 373 to 385 of the JEV E protein may form the neutralization antigen of JEV. However, peptide D, representing amino acids 303 to 400, of the JEV E protein induced only low JEV-neutralizing activity. It would therefore appear that although the sequence of the peptide would certainly be relevant, its conformation would also be important for inducing biologically relevant antibodies.

The 27-amino-acid peptide A contains RGD (Arg-Gly-Asp) sequence that has been reported to be responsible for the cell attachment-promoting activity of the extracellular glycoprotein fibronectin (19). The RGD sequence has also been shown to be involved in foot-and-mouth disease virus cell attachment and initiation of infection (2, 13). The region surrounding the tripeptide RGD in Murray Valley encephalitis virus envelope protein has been suggested to be involved in the virus binding with putative receptor (11). It should be noted that Murray Valley encephalitis virus belongs to same subgroup of the *Flaviviridae* family of animal viruses as does JEV.

The 27-amino-acid peptide A fused to glutathione *S*-transferase was previously shown to induce JEV-neutralizing antibodies in mice (28). However, the authors of that study used a strong adjuvant (Titer Max) during the immunizations. It should be noted that this adjuvant is not permitted for human use. Thus, it is significant that we have succeeded in generating a JEV-neutralizing response with peptide A without the use of an adjuvant. Although the neutralizing antibody titers were low, these imparted protection to immunized mice against a lethal JEV challenge. It should be noted that JEV-neutralizing titers of 1:10 are usually accepted as evidence of protection in humans (34).

Neutralizing antibodies alone are sufficient to impart protection to the vaccinee against JEV. A peptide-based vaccine against JEV is therefore a distinct possibility. The maximum neutralization titer obtained here with CP-A was 1:40; this titer was significantly lower than the titer of 1:600 obtained with a plasmid DNA-based immunization or the titer of more than 1:1,000 obtained with the formalin-inactivated vaccine (8). This may perhaps be due to the likelihood of peptide A forming only a part of the JEV neutralization antigen that may comprise several different epitopes constituted by different peptides. Thus, the success of a peptide-based JEV vaccine would depend upon finding a combination of peptides that would generate high titers of JEV-neutralizing antibodies. It is often argued that empirical rather than rational design is critical for developing a peptide-based vaccine (23). We are therefore examining the immunization potential of a number of other peptide sequences derived from the JEV E protein. The JGMV VLP system can then be used to present several different JEV peptides by mixing different CP fusion proteins and then allowing them to reassemble to form VLPs (6). Another possibility lies in the identification of the mimotopes mimicking the JEV neutralization antigen (35). The JGMV peptide presentation system could then be used to present the mimotopes to raise a high-titer anti-JEV neutralizing immune response.

Several antigen presentation systems, such as the yeast Ty (4)-, HBc (21)-, and bluetongue virus (3)-based systems, are currently under development. The JGMV system has some potential advantages. Since both the N- and the C-terminal regions of JGMV CP are not required for its autoassembly (30), the system could be used for the presentation of appropriate B- and T-cell epitopes simultaneously. Moreover, genetically engineered JGMV RNA, containing gene for the coat protein fused to desired foreign epitopes, could be used to produce virus particles in plants. Such plant material can potentially be used for the oral delivery of the vaccine.

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